

EXHIBIT C

Temporally Distinct and Ligand-Specific Recruitment of Nuclear Receptor-Interacting Peptides and Cofactors to Subnuclear Domains Containing the Estrogen Receptor

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Ligand binding to estrogen receptor (ER) is presumed to regulate the type and timing of ER interactions with different cofactors. Using fluorescence microscopy in living cells, we characterized the recruitment of five different green fluorescent protein (GFP)-labeled ER-interacting peptides to the distinct subnuclear compartment occupied by blue fluorescent protein (BFP)-labeled ER α . Different ligands promoted the recruitment of different peptides. One peptide was recruited in response to estradiol (E₂), tamoxifen, raloxifene, or ICI 182,780 incubation whereas other peptides were recruited specifically by E₂ or tamoxifen. Peptides containing different sequences surrounding the ER-interacting motif LXXLL were recruited with different time courses after E₂ addition. Complex temporal kinetics also were observed for recruitment of the full-length, ER cofactor glucocorticoid receptor-interacting protein 1 (GRIP1); rapid, E₂-dependent recruitment of GRIP1 was blocked by mutation of the GRIP1 LXXLL motifs to LXXAA whereas slower E₂ recruitment persisted for the GRIP1 LXXAA mutant. This suggested the presence of multiple, temporally distinct GRIP1 recruitment mechanisms. E₂ recruitment of GRIP1 and LXXLL peptides was blocked by coincubation with excess ICI 182,780. In contrast, preformed E₂/ER/GRIP1 and E₂/ER/

LXXAA complexes were resistant to subsequent ICI 182,780 addition whereas ICI 182,780 dispersed preformed complexes containing the GRIP1 LXXAA mutant. This suggested that E₂-induced LXXLL binding altered subsequent ligand/ER interactions. Thus, alternative, ligand-selective recruitment and dissociation mechanisms with distinct temporal sequences are available for ER α action *in vivo*. (*Molecular Endocrinology* 14: 2024–2039, 2000)

INTRODUCTION

Ligand binding to ER alters ER conformation and thereby affects its interactions with cofactors regulating gene expression (1–3). Many cofactors that interact specifically with the estradiol (E₂)-bound ER or other ligand-bound nuclear receptors contain one or more copies of the consensus sequence LXXLL (4). Mutation of the LXXLL motifs abrogates ligand-dependent cofactor binding to the ligand-binding domain of many nuclear receptors (4, 5). Peptides containing the LXXLL motifs are themselves sufficient to bind to nuclear receptors (6, 7). Structural studies showed that the LXXLL peptides form an amphipathic α -helix, of which the hydrophobic surface fits into a hydrophobic cleft that forms on the surface of the ER ligand-binding domain in response to E₂ binding (6, 7). The ligand-induced hydrophobic cleft is conserved in the ligand

binding domain of most nuclear receptors (8) and is required for ligand-activated transcription via activation function-2 (AF-2) (9).

The hydrophobic cleft does not form properly upon ER binding to tamoxifen or raloxifene (7, 10), which may account for the antiestrogenic action of these ligands in some tissues. Conversely, tamoxifen and raloxifene have estrogenic effects on other tissues. Like estrogens, tamoxifen and raloxifene promote interaction of some cofactors or peptides with ER structures outside of the hydrophobic cleft (11–14). These interactions probably contribute to the AF-2-independent estrogenic actions of tamoxifen and raloxifene. Novel ER ligands that possess estrogenic activities in most tissues and antiestrogenic activities in the breast and uterus will be clinically useful for reducing the estrogen-mediated increase in breast and endometrial tumors that accompanies otherwise beneficial postmenopausal hormone replacement therapies (15–19). Identification of such improved selective estrogen receptor modulators (SERMs) will be aided by the development of techniques that discern the effects of each putative SERM on the types and timing of ER interactions with ligand-selective ER-interacting targets.

Previously we used phage display to isolate a large number of peptides that bound to different sites on nuclear receptors including ER α (12–14). Each peptide differed in their interactions with specific nuclear receptors or in response to different ligands. Some of the nuclear receptor-interacting peptides contained the LXXLL motif and could be grouped into three classes based upon sequence conservation of the two amino acids immediately amino terminal to LXXLL (12). All three classes of LXXLL are naturally found in cofactors that interact with AF-2. Some cofactors contain multiple LXXLL motifs predominantly of a single class. Others contain LXXLL motifs of varying classes and even LXXLL motifs that are distinct from these three classes. It is thought that such divergence in LXXLL sequence (5, 6, 20), combined with nuclear receptor- or ligand-specific divergences in the structure of the hydrophobic activation function-2 cleft (21), and variations in the interactions of cofactors to other nuclear receptor surfaces, contributes to the divergent actions of different ligands and nuclear receptors.

Although the molecular alterations that accompany ligand binding to nuclear receptors have been intensely characterized (1–3), very little is known of the specificity and order of those events within living cells. Recent studies of fluorophore-labeled nuclear receptors and their interacting cofactors (22–27) demonstrated that the temporal and spatial characteristics of nuclear receptors could be directly examined within cells by fluorescence microscopy. Here, we used fluorescence microscopy to measure in intact cells the ligand-specific interactions of ER with the nuclear receptor cofactor GRIP1 and five peptides that we re-

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cently selected from combinatorial libraries for their binding to ligand-bound ER (12–14). Human ER α expressed as a fusion with blue fluorescent protein (28) (BFP) localized to discrete subdomains of the nucleus. GRIP1 (glucocorticoid receptor-interacting protein 1) and the peptides expressed in cells as fusions with the spectrally distinct green fluorescent protein (28) (GFP) were more evenly distributed throughout the nucleus; the GFP-peptide fusions were also present in the cytoplasm. When coexpressed with ER α -BFP in cells not treated with ER ligand, the GFP-peptides and GFP-GRIP1 exhibited the same distributions as when expressed alone. When incubated with E₂, three peptides containing variants of LXXLL relocated to assume the intranuclear position of ER. A fourth, unrelated peptide was selectively recruited in response to tamoxifen whereas recruitment of a fifth peptide was promoted by any of E₂, tamoxifen, raloxifene, or the antiestrogen ICI 182,780. GRIP1 was selectively recruited by E₂ or tamoxifen incubation. Simultaneous incubation with an excess of ICI 182,780 blocked recruitment of GRIP1, each LXXLL peptide, and the tamoxifen-specific peptide.

Recruitment of the peptides and GRIP1 to the intranuclear location of ER α in living cells mimicked their previously reported ligand dependence and efficacy of ER α interaction. In addition to confirming in living cells the ligand specificities of these interactions, the intranuclear recruitment assay uniquely enabled us to determine that each peptide and GRIP1 varied in the timing of recruitment after ligand addition. Surprisingly, temporal studies of dissociation showed that preformed complexes involving LXXLL interactions with ER uniquely were not disrupted even after 4 h of incubation with a 1,000-fold molar excess of ICI 182,780. Thus, we report a novel procedure for investigating the ligand-specific recruitment of labeled factors or peptides to nuclear receptors in living cells. This allowed us to determine the unique timing of different ligand-specific complexes formed with ER and to discover that LXXLL-dependent interactions alter the availability of the receptor for subsequent ligand binding in living cells.

RESULTS

E₂-Dependent Relocalization of Class I, II, and III LXXLL Peptides to the Intranuclear Subcompartment Containing ER α

We previously isolated three different classes of LXXLL-containing, ER α -interacting peptides by phage display (12–14). All three classes are represented in known ER α -interacting cofactors, including a receptor-interacting protein of 140 kDa (RIP140) (29), a thyroid hormone receptor accessory protein of 220 kDa (TRAP₂₂₀) (30), a vitamin D receptor-interacting protein of 205 kDa (DRIP₂₀₅) (31), and the homologous coac-

tivators glucocorticoid receptor-interacting protein (GRIP1) (32, 33), and steroid receptor coactivator (SRC-1a)(34). For instance, RIP140 contains 11 LXXLL motifs, eight of which are of the class III type (S/T, Φ ,LXXLL where Φ is any hydrophobic amino acid) whereas TRAP₂₂₀ and DRIP₂₀₅ each contain two LXXLL motifs, both of the class II type (P, Φ ,LXXLL). GRIP1 and SRC-1a have, in common, three divergent LXXLL motifs, the most amino terminal of which is of the class I type (S/T, K/R, LXXLL), and two more carboxy-terminal LXXLL motifs that do not readily fit into any of the three classes.

Oligonucleotides encoding peptide sequences representative of each of the class I, II, and III peptides were fused in frame to the carboxy terminus of GFP (see Fig. 1) and expressed in mouse GHFT1-5 cells. The intracellular locations of GFP and each GFP-labeled LXXLL peptide were identified by fluorescence microscopy after their expression. GFP (not shown) and the three GFP-LXXLL fusions were distributed throughout the cytoplasm and nucleus (Fig. 2, A–C, left panels). The proportion of GFP-LXXLL fluorescence in the nucleus and cytoplasm varied from evenly distributed between nucleus and cytoplasm to some

nuclear preference. The variation in nuclear/cytoplasmic partitioning was independent of expression level and was globally similar for GFP and each GFP-LXXLL fusion.

To determine whether ER α expression altered the distribution of GFP-LXXLL, ER α was coexpressed as an in-frame fusion with BFP. This allowed us to separately track the locations of ER α -BFP and GFP-LXXLL in the same cell by selectively exciting and detecting their corresponding blue and green emissions (35). The ER α -BFP fusion was functional as, like native ER α (36), it cooperated with the transcription factor Pit-1 to activate the PRL promoter in GHFT1-5 cells (not shown). The ER α -BFP fusion also activated the transcription of a minimal promoter under the control of an isolated ER binding site in HeLa and DU145 cells (not shown). In contrast to the cytoplasmic and uniform intranuclear distributions of GFP-LXXLL, ER α -BFP was exclusively nuclear and assumed a reticular pattern of distribution within the nucleus (Fig. 2, ER α -BFP). This reticular intranuclear distribution has been previously reported for ER (22, 27) as well as other nuclear receptors (23–26) and is more pronounced

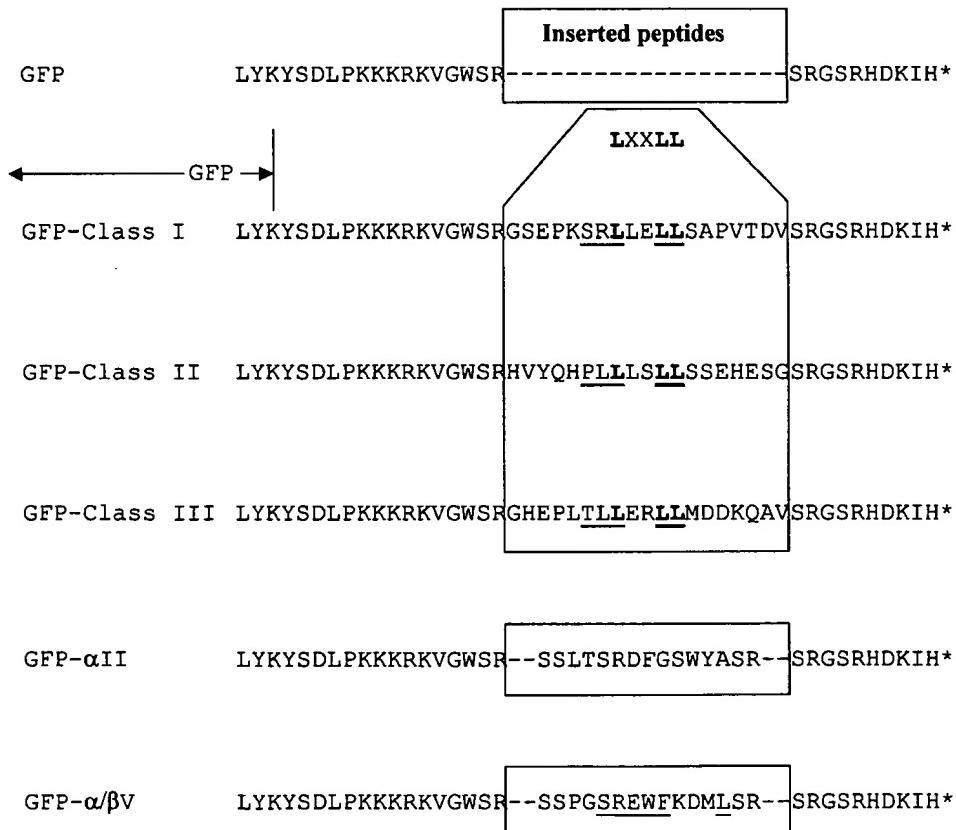


Fig. 1. ER-Interacting Peptides Fused to the Carboxy Terminus of GFP

Box represents sequence of ER-interacting peptides isolated in Chang *et al.* (12) and Norris *et al.* (13). Underlined amino acids in peptide sequence are those conserved in the class I, class II, and class III LXXLL peptides (12). Underlined in the α/β V peptide are those amino acids conserved in other isolated peptides and in receptor potentiating factor 1 (13). *, Carboxy terminus of fusion proteins. The spacer between GFP and the ER-interacting peptide sequence contains the Simian Virus 40 NLS that, because of the small size of GFP, did not have much effect on nuclear localization.

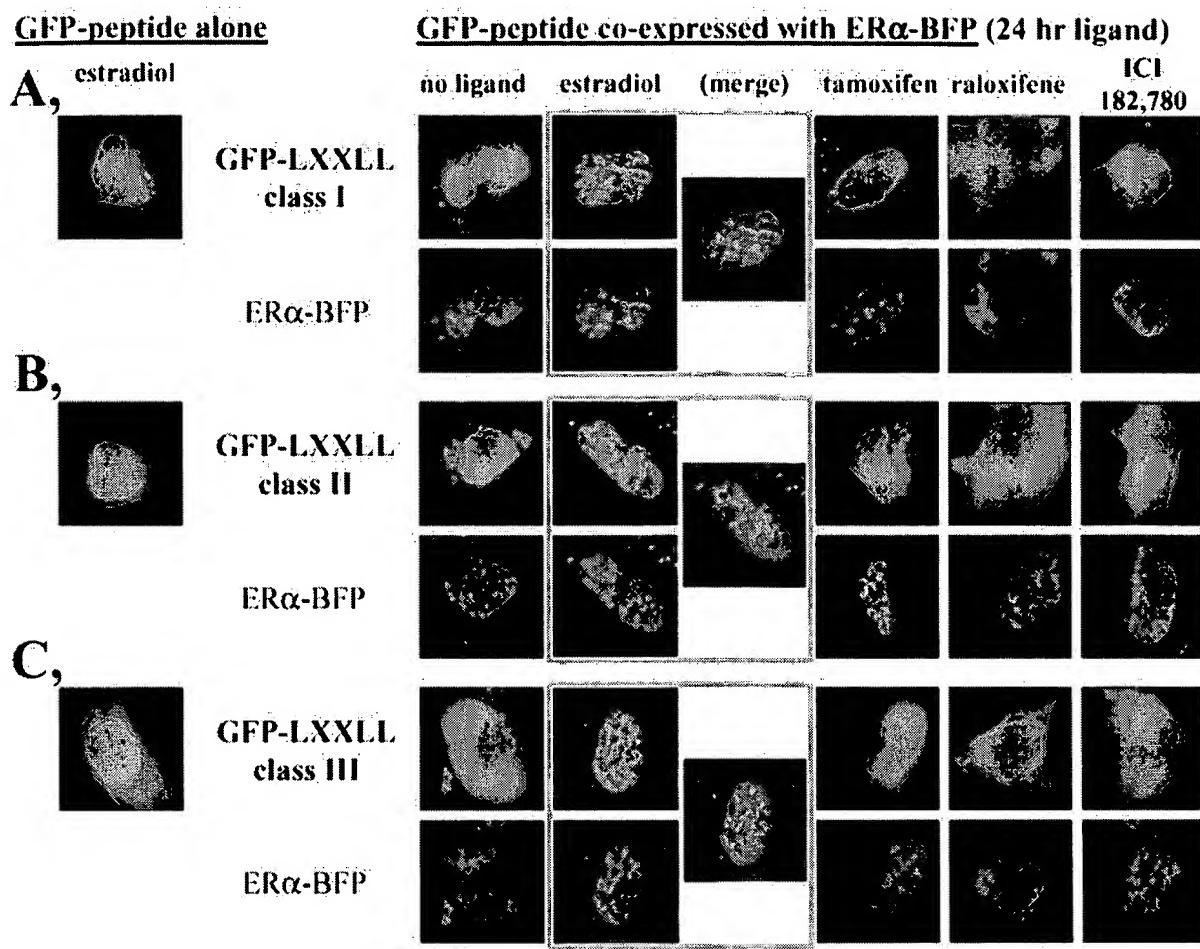


Fig. 2. E_2 -Dependent Recruitment of GFP Fused to Class I, Class II, and Class III LXXLL Peptides to the Intranuclear Location of ER α -BFP

Fluorescence microscopic detection of the intracellular localization of the three GFP-LXXLL fusions (A–C) coexpressed with ER α -BFP in cells. One day after transfection, cells were treated either with ethanol vehicle (no ligand), 10^{-8} M estradiol, 10^{-6} M tamoxifen, 10^{-7} M raloxifene or 10^{-7} M ICI 182,780. One day after ligand addition, green and blue fluorescence was selectively detected by fluorescence microscopy as described in *Materials and Methods*. *Merge*, Merged blue and green images of the cells incubated with E_2 indicate overlap in the intranuclear location of ER α -BFP and each GFP peptide as a cyan coloration. Green fluorescence from each GFP-peptide fusion in the absence of ER α -BFP expression is shown from cells incubated with 10^{-8} M estradiol (*left panels*).

when the cells are incubated with E_2 or SERMs (22, 27).

In cells grown in E_2 -free media, the dispersed cellular distribution of each GFP-LXXLL fusion was unchanged upon coexpression of ER α -BFP (Fig. 2, A–C, no ligand). In contrast, incubation of cells coexpressing ER α -BFP and any of the GFP-LXXLL fusions with 10^{-8} M E_2 caused the GFP-LXXLL to assume the reticular pattern characteristic of ER α -BFP in the nucleus (Fig. 2, A–C, estradiol). Complete overlap of GFP-LXXLL with ER α -BFP in the identical subnuclear compartment after E_2 addition is indicated by the exclusively cyan-colored image obtained when the separate blue and green images are merged (Fig. 2, A–C, merge). This was observed in cells that express GFP-LXXLL in low stoichiometry relative to ER α -BFP. In cells expressing more GFP-LXXLL than ER α -BFP, co-

localization of GFP-LXXLL and ER α -BFP was observed as a concentration of green fluorescence at the site of blue fluorescence (not shown). When ER α -BFP was not coexpressed, there was no intranuclear redistribution of GFP-LXXLL in the presence of E_2 (Fig. 2, A–C, *left panels*) or any other ER ligand (not shown). Similarly, GFP itself did not redistribute to ER α -BFP upon incubation with E_2 or any other ER ligand (not shown). Thus, relocalization of GFP-LXXLL was specifically dependent upon the LXXLL peptide, coexpression of ER α -BFP, and addition of E_2 .

Intracellular Relocalization of Different LXXLLs to ER α Parallels Their Interaction Profiles

To further characterize the ligand dependence of GFP-LXXLL colocalization with ER α -BFP, we determined

the E₂-induced relocalization kinetics of each of the class I, class II, and class III GFP-LXXLLs to ERα. Each GFP-LXXLL was coexpressed with ERα-BFP in cells grown in E₂-free media. One day after transfection, parallel coverslips were incubated with no hormone, or with 10⁻¹⁰, 10⁻⁹, 10⁻⁸ or 10⁻⁷ M E₂ for 24 h. We then determined the fraction of cells in which GFP-LXXLL colocalized with ERα-BFP for each E₂ concentration.

By fluorescence microscopy, we scanned the coverglass using blue fluorescence excitation and emission filters to first identify cells expressing ERα-BFP. We then rapidly switched to the green filter set to determine whether the cell contained visible GFP-linked target. If the GFP-linked target was also present, it was then scored as colocalized if there was any concentration of green fluorescence at the site of the ER. By scoring GFP-peptide or cofactor-expressing cells only after determining which cells obviously contained ERα-BFP, we avoided the bias in which a bright, reticular GFP fluorescence pattern would inflate our detection of colocalized cells containing otherwise undetectable levels of the generally less fluorescent ERα-BFP. By setting the colocalization criterion as "any" colocalization, we also removed any biases that would have resulted if we had attempted to subjectively score cells for the variable extent of colocalization. Since the proportion of non-colocalized cells decreases with increasing colocalization, the recruitment of specific factors or peptides is measured as the change in the proportion of cells that responded after the addition of different concentrations of E₂. The validity of this approach was confirmed by the high reproducibility of the data obtained from multiple independent experiments, which are plotted in Fig. 3A as the mean ± SD in the percent of cells showing colocalization at each ligand concentration. Half-maximal binding to ERα-BFP with each class of GFP-LXXLL was reached at 3–7 × 10⁻¹⁰ M E₂, approximately the concentration of E₂ needed for activation of ER-regulated promoters in cell transfection studies (37).

Essentially complete colocalization with ERα-BFP was achieved with 10⁻⁸ M E₂ for both the class I and the class III GFP-LXXLL fusions. In contrast, colocalization of the class II GFP-LXXLL did not increase beyond a maximum of 57 ± 6% of the cells. This limit did not appear to be a function of the level of peptide expressed in the cell as the proportion of cells showing colocalization remained constant over a wide range of GFP-class II LXXLL expression (Fig. 3B). In these studies, expression of GFP-LXXLL was modulated from a tetracycline-inducible promoter by varying the levels of the inducer, doxycycline. Note that all images in Fig. 3B were taken with the same short exposure times that were insufficient to detect the basal expression level of the GFP-LXXLL peptide in the absence of doxycycline. Thus, the observed deficiency in the *in vivo* ERα-BFP interaction of the class II LXXLL relative to the class I and III LXXLLs was not related to differences in the ex-

pression of these peptides. The reduced efficiency of colocalization of the GFP-class II LXXLL with ERα-BFP accurately mimicked the poorer interaction of the class II peptide with ERα that we had previously observed (12).

Delayed Temporal Kinetics of Class II LXXLL Recruitment to ERα

Sequence-specific differences in colocalization of the three LXXLL peptides with ERα were also evident in time course studies. We conducted single cell recordings of the E₂-induced intracellular recruitment of LXXLL to ERα. First, we identified cells, grown in the absence of E₂, that expressed both the class I GFP-LXXLL and ERα fused to red fluorescent protein (RFP). The ERα-RFP fusion protein was functionally active in the ligand-induced activation of estrogen-responsive promoters (data not shown). ERα-RFP and GFP-LXXLL digital images of the same cell were captured using red and green fluorescent filter sets before the addition of ligand and at 1-min intervals after the addition of 10⁻⁶ M E₂. An example of one cell before E₂ addition and 20 min after E₂ addition is provided in Fig. 4A. Appropriate controls, using cells expressing only ERα-RFP or GFP-LXXLL of intensities equivalent to those in the coexpressing cells, showed that there was no fluorescence bleedthrough between the red and green images (not shown).

Only partial colocalization of GFP-LXXLL with ERα-RFP (or ERα-BFP) was obtained after these short incubation periods (see Fig. 4A), which contrasts with the complete overlap of GFP-LXXLL and ERα-RFP (or ERα-BFP) after 24-h incubations (Fig. 2). To quantify partial colocalization at the short time frames, we measured the intensity of green GFP-LXXLL and red ERα-RFP fluorescence for each pixel within the nucleus of each cell image. The intensity of green fluorescence within the nucleus that colocalized with the red fluorescence of ERα-RFP was divided by the intensity of green fluorescence in the regions of the nucleus from which ERα-RFP was less concentrated (defined at those regions of the nucleus in which ERα-RFP intensity was less than 75% of the maximal ERα-RFP intensity). This ratio was calculated before the addition of hormone and at 1-min time intervals after E₂ addition for 15 different single cell recordings. The change in this ratio is plotted over 20 min after E₂ addition in Fig. 4B; a positive change in the pixel intensity ratio indicates that more GFP-LXXLL was concentrating at the intranuclear location of ERα-RFP. Notably, the response for each cell varied from cells displaying relatively random fluctuations of green/red pixel intensities of -0.02 to +0.02 to cells in which there was an obvious concentration of the GFP-LXXLL at the intranuclear location of ERα-RFP over the 20-min time course. For comparison, the read-out obtained from a cell shown in Fig. 4A, in which con-

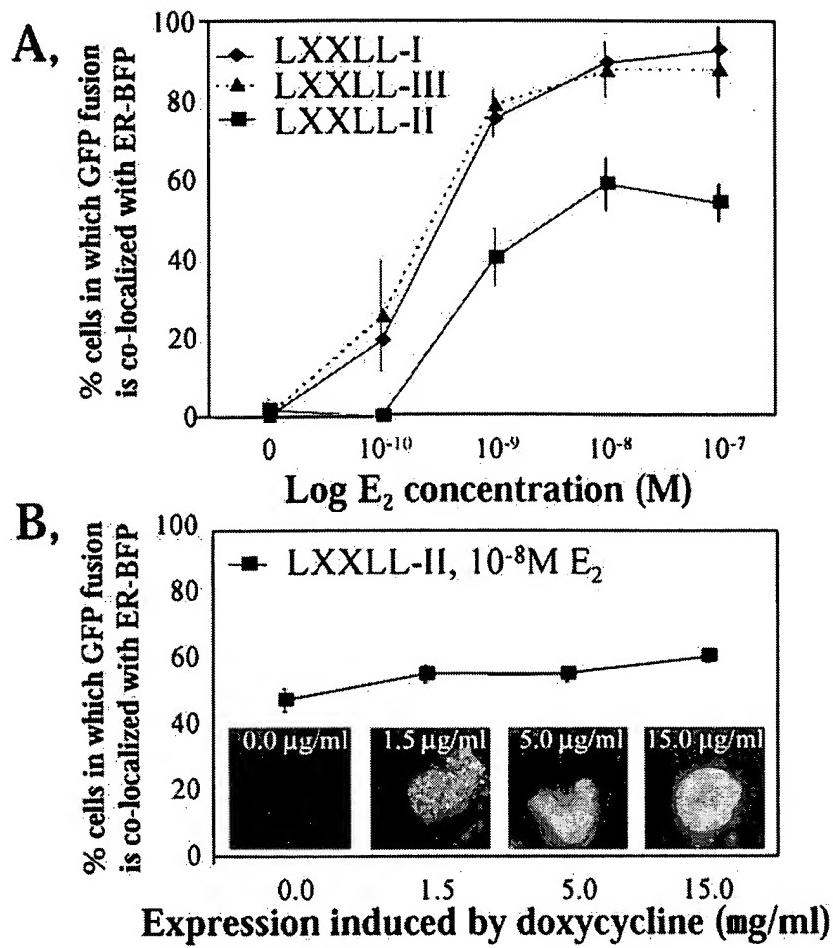


Fig. 3. Different LXXLL-Containing Peptides Are Recruited to ER α with Different Kinetics

A, The proportion of cells in which class I, class II, or class III GFP-LXXLL colocalizes with ER α -BFP increases with increasing E_2 dose. Half-maximal colocalization is achieved at E_2 concentrations required for promoter activation (37, 50). The graph represents the mean \pm SD in the percent of cells displaying colocalization from three independent experiments. B, The poorer colocalization of the class II GFP-LXXLL with ER α -BFP is independent of the expression level of class II GFP-LXXLL. Representative green fluorescence from individual cells shows the different expression levels of class II GFP-LXXLL induced by the indicated concentrations of doxycycline used to drive the linked tetracycline-inducible promoter. In the absence of doxycycline, green fluorescence was visible but not captured with the short exposure times used to collect the images shown. The graph represents the mean \pm SD in the percent of cells displaying colocalization from a single transfection collected three separate times over 1 day.

centration of GFP-LXXLL at the site of ER α -RFP is at the threshold of being visible by the naked eye, is indicated by the open triangles (see Fig. 4B, *). Thus, over short time periods, recruitment of GFP-LXXLL to ER α -RFP, measured quantitatively, is highly variable with time after E_2 addition.

The cell-to-cell variability in recruitment over short time periods required that we score large numbers of cells at each time point to obtain data in which we have confidence. This could not be accomplished by recording individual cells for prolonged time periods. However, our prior experience demonstrated that we could readily and reproducibly score by visual inspection 50–150 cells within a 10-min window (Fig. 3). We coexpressed each GFP-LXXLL together with ER α -BFP in cells grown in the absence of ligand and then

scored those cells, exactly as for Fig. 3, for colocalization in 10-min windows between 15–25 min, 40–50 min, and 85–95 min at 24 h after the addition of 10^{-8} M E_2 (Fig. 4C).

Colocalization of the class I and class III GFP-LXXLLs with ER α -BFP was detected within 20 min after the addition of 10^{-8} M E_2 and increased thereafter. This represented the time required for E_2 to enter the cell, bind to ER α -BFP, and have detectable amounts of freely diffusing GFP-LXXLL concentrate at the intranuclear location of the liganded ER α -BFP. Whereas the colocalization of GFP-class I LXXLL and GFP-class III LXXLL fusions with ER α -BFP showed identical time courses and reached similar levels in response to saturating levels of E_2 , the redistribution of the class II peptide to ER α -BFP was much less rapid (Fig. 4C).

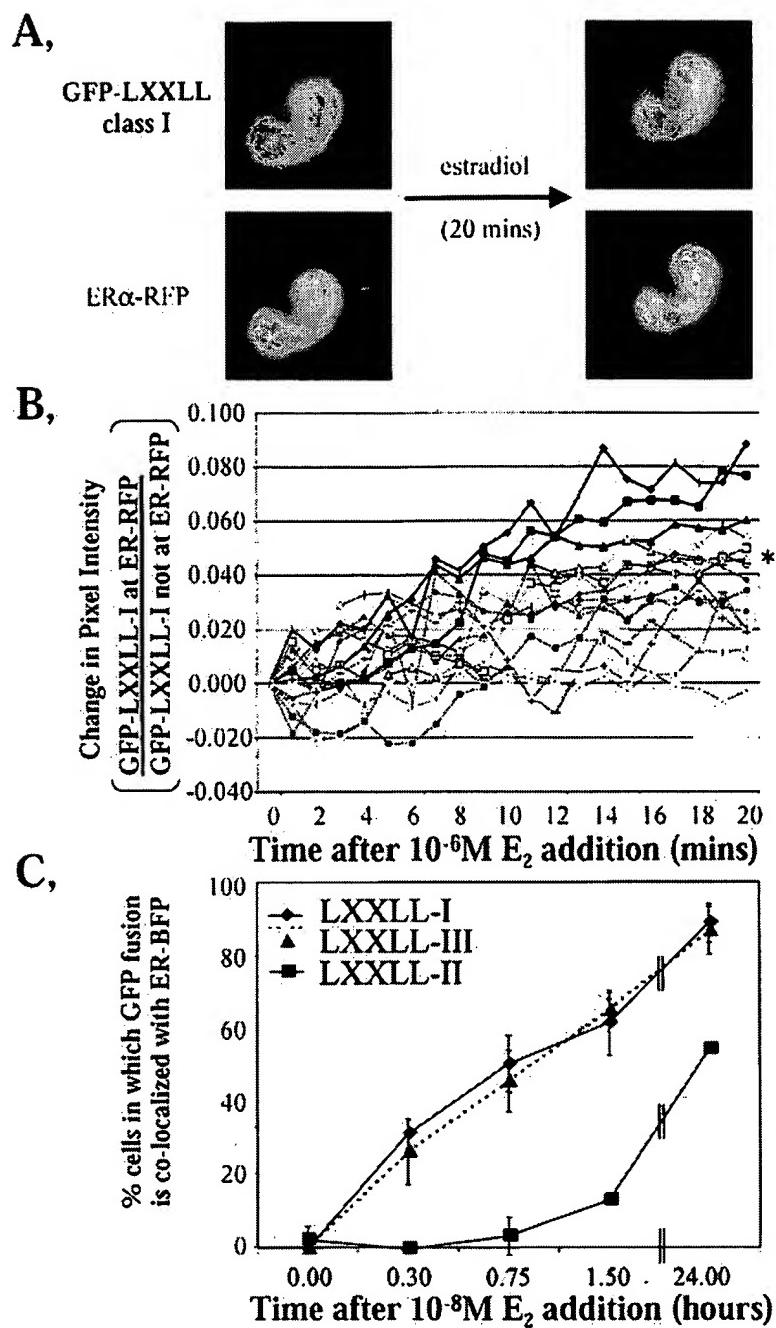


Fig. 4. Different LXXLL-Containing Peptides Are Recruited to ER α with Different Temporal Kinetics

A, Sequential images of the same cell captured before and 20 min after the addition of 10^{-6} M E₂ demonstrate partial colocalization of the class I LXXLL-GFP fusion protein with ER α fused to RFP. B, Quantification of the level of GFP and RFP fluorescence at each pixel within the nucleus at 1-min time intervals after the addition of 10^{-6} M E₂ shows variable concentration of class I LXXLL-GFP at the position of ER α -RFP in 15 different cells. *, Readout of cell shown in Fig. 4A (open triangles). C, The proportion of cells showing any colocalization of ER α -BFP with each GFP-LXXLL was scored with time after addition of 10^{-8} M E₂. The slower time course and reduced binding of the class II peptide reflect the previously reported poorer interaction of this peptide with ER α (12). Each graph represents the mean \pm SD deviation in the percent of cells displaying colocalization from three independent experiments.

The delayed time course of class II LXXLL intracellular colocalization with ER α -BFP, which would not have been detected in other assays, demonstrated that ER association with different LXXLL sequences follows

different temporal kinetics. An intriguing possibility is that the different LXXLL temporal kinetics underlies a previously proposed (38) sequential recruitment of co-factors to ER after E₂ addition.

Table 1. Ligand-Specific Complexes Formed by ER α and Indicated Peptides or GRIP1

Ligand : (24 hour incubation)	no ligand	ICl 182,780 5×10^{-7} M	Raloxifene 5×10^{-7} M	Tamoxifen 5×10^{-7} M	Estradiol 5×10^{-8} M	ICl inhibition $E_2, 10^{-9}$ M or Tam, 10^{-7} M to $\alpha/\beta V$	+ ICl, 10^{-6} M
GFP -							
LXXLL -I	2 +/- 2%	0 +/- 0%	0 +/- 0%	21 +/- 9%	93 +/- 6%	79 +/- 7% 	0 +/- 0%
LXXLL -II	2 +/- 2%	1 +/- 1%	1 +/- 1%	3 +/- 2%	64 +/- 17%	47 +/- 4% 	0 +/- 0%
LXXLL -III	0 +/- 0%	*	*	*	90 +/- 8%	76 +/- 4% 	3 +/- 1%
α II	0 +/- 0%	85 +/- 8%	46 +/- 5%	76 +/- 1%	56 +/- 2%	32 +/- 11% 	84
$\alpha/\beta V$	0 +/- 0%	0 +/- 0%	2 +/- 2%	81 +/- 14%	2 +/- 2%	52 +/- 10% 	1 +/- 0%
GRIP1 wt	9 +/- 5%	3 +/- 3%	4 +/- 3%	21 +/- 12%	81 +/- 17%	63 +/- 17% 	6 +/- 3%
GRIP1 Δ LXXLL	6 +/- 3%	3 +/- 3%	0 +/- 0%	5 +/- 4%	48 +/- 6%	43 +/- 5% 	3 +/- 1%

* , no co-localization in some LXXLL-III data not quantified by cell counting
shading represents indicated % co-localization with ER α

 X%
 X%
 X%
 X%
 X%

0-9% 10-34% 35-59% 60-89% 90-100%

Ligand-Specific Differences in Class I, II, and III LXXLL Colocalization with ER α

Incubation of cells coexpressing GFP-LXXLL and ER α -BFP overnight with 10^{-6} M tamoxifen resulted in a slight concentration of green fluorescence emitted from the class I GFP-LXXLL over the reticular pattern of ER α -BFP fluorescence (Fig. 2A). This weak colocalization was reproducible and was quantified in Table 1 as the percentage of cells in which the indicated GFP-linked peptide showed any visible colocalization with ER α -BFP in response to the indicated ER ligand. Tamoxifen promoted class I GFP-LXXLL colocalization with ER α -BFP but not class III GFP-LXXLL colocalization with ER α -BFP (Table 1). Thus, the class I and class III peptides, which behaved identically in response to E₂ (Figs. 3A and 4C), differed in their response to tamoxifen. The class II GFP-LXXLL also did not appreciably respond to tamoxifen (Table 1).

In contrast to tamoxifen, incubating the cells overnight with two other SERMs, raloxifene or ICI 182,780, did not promote overlap in the intracellular distributions of coexpressed ER α -BFP and any of the class I, II, or III GFP-LXXLLs (Table 1). Raloxifene and ICI 182,780 were effective in promoting the colocalization of another, unrelated peptide with ER α -BFP (Fig. 5A). This peptide, α II, was previously selected for the ability to interact with ER α bound by either E₂ or tamoxifen (13). In the absence of ligand, the intracellular distribution of GFP- α II was diffuse (Fig. 5A). However, E₂, tamoxifen, raloxifene, and ICI 182,780 each induced GFP- α II to redistribute to the intranuclear location occupied by ER α -BFP (Fig. 5A and Table 1). Relocalization was specifically dependent upon the α II peptide, ER α -BFP coexpression, and ligand addition. Thus, E₂, tamoxifen, raloxifene, and ICI 182,780 were all capable of entering the cell and promoting specific ER α -peptide interactions. The different ligand specificities of

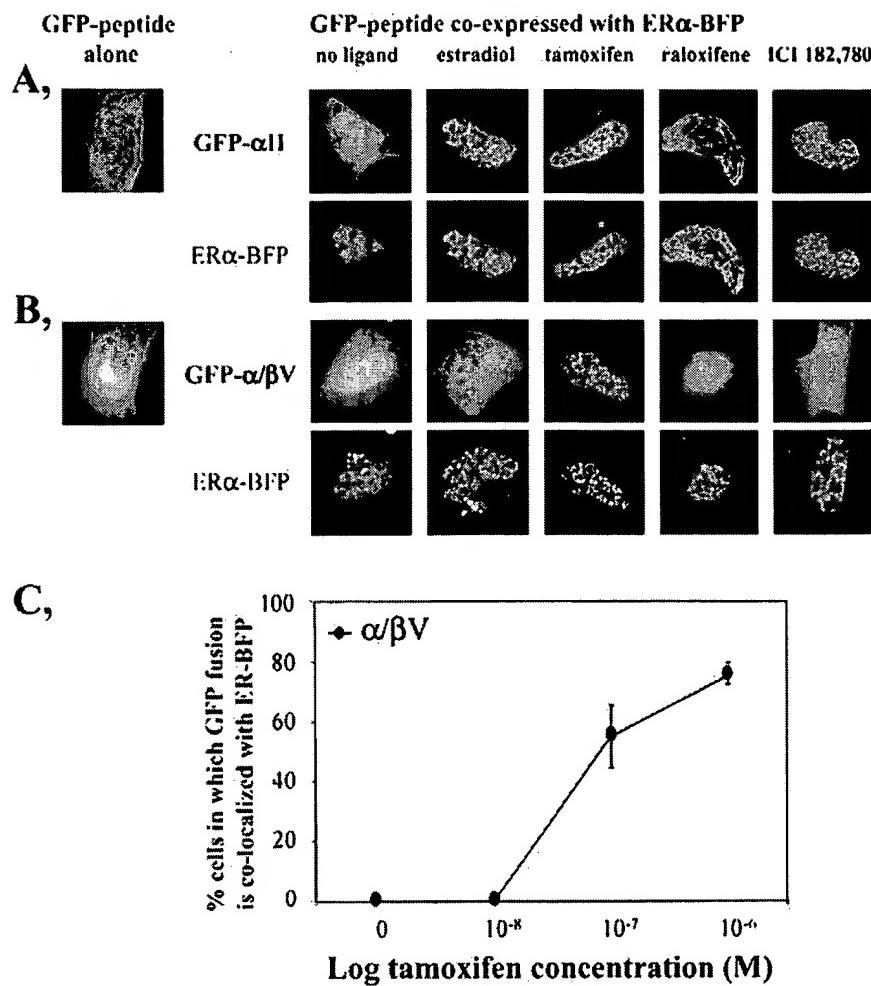


Fig. 5. Different Ligand Dependence of the Recruitment of non-LXXLL-Containing, ER-Interacting Peptides to the Intranuclear Location of ER α

A, Intracellular recruitment of GFP fused to the αII peptide (13) to ER α -BFP is induced by incubating the cells with any of E₂, tamoxifen, raloxifene, or ICI 182,780. B, Tamoxifen-dependent, intranuclear colocalization of ER α -BFP and GFP fused to the $\alpha/\beta V$ peptide (13). Ligand incubation and fluorescence detection are as described in the legend to Fig. 2. Green fluorescence from each GFP-peptide fusion in the absence of ER α -BFP expression (left panels) is shown from cells incubated with 10⁻⁸ M E₂ (A) or 10⁻⁶ M tamoxifen (B). C, Half-maximal recruitment of GFP- $\alpha/\beta V$ to ER α -BFP is achieved at tamoxifen concentrations that parallel tamoxifen induction of ER α promoter activation (13). The graph represents the mean \pm SD in the percent of cells displaying colocalization from three independent experiments.

the LXXLL and αII peptide interactions demonstrate the subtle differences in receptor conformation promoted by each ligand.

Tamoxifen-Specific Colocalization of an ER-Interacting Peptide with ER α -BFP

SERM-selective *in vivo* recruitment also was shown by the tamoxifen-specific recruitment of another peptide, $\alpha/\beta V$, selected previously from a combinatorial library for interaction only with ER α bound to tamoxifen (13). We fused the $\alpha/\beta V$ peptide in frame to GFP (Fig. 1) and determined that, like GFP, GFP-LXXLL, and GFP- αII , the GFP- $\alpha/\beta V$ fusion distributed throughout the nucleus and was present to varying degrees in the cyto-

plasm (Fig. 5B). In cells not expressing ER α -BFP, GFP- $\alpha/\beta V$ remained distributed after treatment with tamoxifen (Fig. 5B, left panel) or any other ligand. Similarly, when coexpressed with ER α -BFP in cells grown in the absence of ligand or after incubation with 10⁻⁸ M E₂, 10⁻⁷ M raloxifene, or 10⁻⁷ M ICI 182,780, the subcellular localization of GFP- $\alpha/\beta V$ was not altered. In contrast, incubation with 10⁻⁶ M tamoxifen resulted in a concentration of GFP- $\alpha/\beta V$ at the intranuclear reticular pattern characteristic of ER α -BFP. Tamoxifen dose-response curves (Fig. 5C) showed that GFP- $\alpha/\beta V$ relocation to ER α -BFP corresponded with the promoter activation profile of tamoxifen bound to ER α (37). Tamoxifen-selective GFP- $\alpha/\beta V$ colocalization with ER α -BFP demonstrated that ER α

adopts a conformation *in vivo* that is different than that adopted by the E₂-, raloxifene-, or ICI 182,780-bound receptors.

Ligand-Dependent Recruitment of the Full-Length ER-Interacting Cofactor GRIP1 to the Intranuclear Compartment Containing ER α

The ligand-specific and temporally distinct associations of different ER-interacting peptides with ER α suggested that a similar approach could be employed to demonstrate the ligand specificity and pattern of recruitment of full-length ER-interacting cofactors to ER α *in vivo*. Indeed, one ER coactivator SRC-1a fused to GFP recently was shown to be recruited to the intranuclear location of ER α fused to the cyan fluorescent protein upon E₂, but not tamoxifen or ICI 182,780, incubation (27). We determined that the related ER-interacting cofactor GRIP1, fused to GFP, was also recruited upon ligand addition to the intracellular subcompartment containing ER α -BFP (Fig. 6) and then detailed the ligand specificity and kinetics of that recruitment (Fig. 7).

GFP-GRIP1, when expressed by itself, was exclusively nuclear and dispersed throughout the intranu-

clear compartment, although absent from nucleoli (Fig. 6A). In cells coexpressing GFP-GRIP1 and ER α -BFP and incubated with ICI 182,780 or raloxifene, GFP-GRIP1 retained its characteristic dispersed distribution (Fig. 6B). In contrast, both E₂ and tamoxifen were very effective in recruiting GFP-GRIP1 to ER α -BFP (Fig. 6B and Table 1). This again illustrated that E₂ and each SERM promote very distinct ER interactions with specific ER-interacting factors and motifs.

Temporal Variation in LXXLL Requirements for E₂ and Tamoxifen Recruitment of GRIP1 to ER α

E₂ and tamoxifen-specific recruitment of GFP-GRIP1 to ER α -BFP was distinguished by their different time courses and dependencies upon the LXXLL motifs in GRIP1 (Fig. 7). E₂ recruitment was characterized by a rapid LXXLL-dependent phase followed by a slow LXXLL-independent phase. Rapid recruitment was detected as an initial plateau of 30–40% of the cells displaying colocalization of GFP-GRIP1 with ER α -BFP within 20 min after E₂ addition (Fig. 7A, GRIP1-wt). This early phase plateau was blocked (Fig. 7A, GRIP1-ΔLXXLL) by mutation to LXXAA of the two LXXLL motifs of GRIP1 required for interaction with the ER

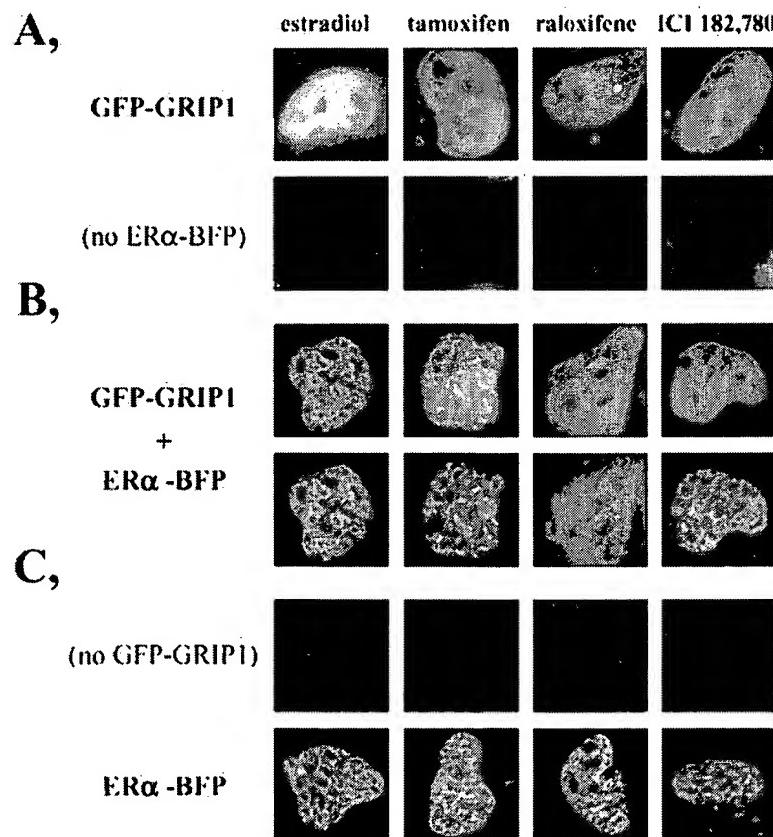


Fig. 6. Recruitment of GRIP1 to the Intranuclear Location of ER α Is Ligand-Selective

A, In the absence of coexpressed ER α -BFP, GRIP1 is evenly distributed throughout the nucleoplasm but is absent from nucleoli. B, When coexpressed with ER α -BFP, GFP-GRIP1 assumes the intranuclear location of ER α -BFP only if incubated with E₂ or tamoxifen. C, Cells expressing ER α -BFP only. Ligand incubation and fluorescence detection are as described in the legend to Fig. 2.

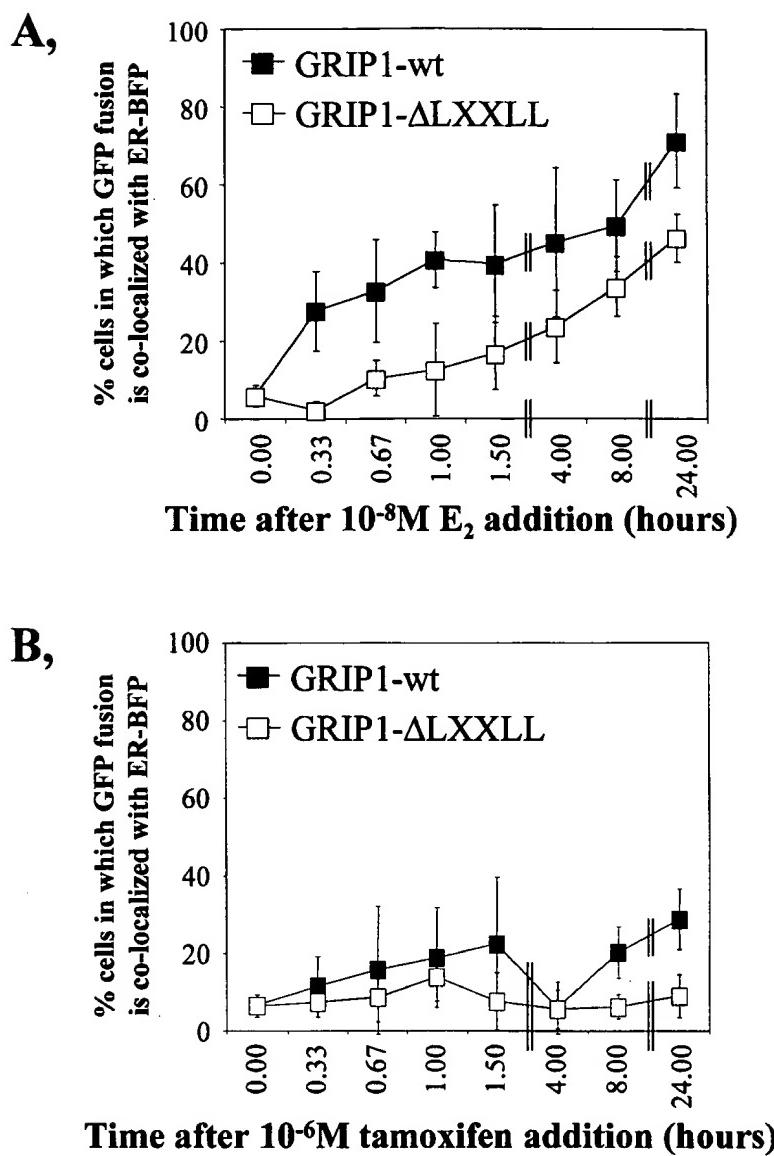


Fig. 7. Different Temporal Kinetics for LXXLL-Dependent and LXXLL-Independent Recruitment of GFP-GRIP to ER α -BFP
A, The proportion of cells in which GRIP1 colocalizes with ER α -BFP increases rapidly with time after E_2 addition. Rapid colocalization is lost if the second and third LXXLL motifs of GRIP1 are mutated to LXXAA (GRIP1- Δ LXXLL). GRIP1- Δ LXXLL is still induced by E_2 to colocalize with ER α but with much reduced temporal kinetics indicating that two separate, and temporally distinct mechanisms govern E_2 -dependent, intranuclear recruitment of GRIP1 to ER α . **B,** Recruitment of GRIP1 to the intranuclear location of ER α is LXXLL-dependent at 8 h or more after tamoxifen addition. Recruitment at earlier time points is variable, but not statistically significant. Each graph represents the mean \pm SD in the percent of cells displaying colocalization from three independent experiments.

ligand binding domain (5). A more gradual increase in GRIP1/ER α colocalization that followed 1.5 h after E_2 addition was not abrogated by the LXXAA mutations. These complex temporal kinetics and LXXLL dependencies suggest time-dependent variations in the available types of E_2 /ER/GRIP1 associations with some lagging associations possibly dependent upon interim interactions and/or enzymatic processes.

The weaker tamoxifen-dependent recruitment of GFP-GRIP1 to ER α -BFP also displayed a complex time course (Fig. 7B) that was mechanistically distinct

from that induced by E_2 . Tamoxifen-induced colocalization of GFP-GRIP1 and ER α -BFP was statistically significant at 8 and 24 h after tamoxifen addition. Before that, a slow gradual recruitment of GRIP1 was not statistically significant. A precipitous drop in colocalization at 4 h after tamoxifen addition may indicate some tendency toward a temporally biphasic response, but this interpretation is questionable given that the change in colocalization at the early time points was not statistically significant. The statistically significant tamoxifen-dependent colocalization at 8

and 24 h was disrupted by the mutation of the GRIP1 LXXLL motifs to LXXAA (Fig. 7B). Because it is unlikely that the LXXLL motifs of GRIP1 interact directly with the tamoxifen-bound ER, the LXXLL dependence of the slow, tamoxifen-dependent GRIP1 recruitment may reflect a more indirect recruitment of GRIP1 to the tamoxifen-bound ER or a dependence on additional motifs present in GRIP1.

E₂/ER α /LXXLL Complexes Become Resistant to Subsequent Challenge with Antiestrogen

Previous reports showed that ligand-induced binding of LXXLL to ER *in vitro* caused an alteration in the rate by which the ligand dissociates from the ER (39). To determine whether LXXLL interaction with ER *in vivo* might similarly slow ligand access to ER, we examined whether ER α /cofactor or ER α /peptide complex formation altered access to the antiestrogen ICI 182,780. ICI 182,780 did not promote ER α -BFP colocalization with GFP-GRIP1, GFP- α / β V, or any of the three GFP-LXXLL fusions (Table 1). Consistent with its role as an antiestrogen, simultaneous addition of 10⁻⁶ M ICI 182,780 with 10⁻⁹ M E₂ abrogated colocalization of ER α -BFP with each of the three GFP-LXXLL fusions and GFP-GRIP1 (Table 1, ICI inhibition) whereas colocalization of the ICI 182,780-responsive, all peptide was unaffected. ICI 182,780 (10⁻⁶ M) also blocked colocalization of ER α -BFP and GFP- α / β V in response to 10⁻⁷ M tamoxifen (Table 1).

Having showed that 10⁻⁶ M ICI 182,780 effectively blocked recruitment of GRIP1 and the α / β V, LXXLL-I, LXXLL-II and LXXLL-III peptides to ER α , we next determined the temporal kinetics of complex dissociation. To do so, we challenged preformed complexes with an excess of ICI 182,780 to block the reformation of transiently dissociated complexes. Initially, the proportion of cells containing ER α colocalized with GFP- α / β V after 24 h incubation with 10⁻⁷ M tamoxifen was determined, 10⁻⁶ M ICI 182,780 was added to the media, and the cells subsequently were scored for any colocalization at the indicated time points after ICI 182,780 addition (Fig. 8A). Complete disruption of colocalization of GFP- α / β V and ER α -BFP was observed within 2 h of ICI 182,780 addition. This established that 2 h was sufficient time for ICI 182,780 to enter the cells, disrupt all preformed complexes, and completely release all GFP- α / β V concentrated at the ER α subcompartment.

For complexes of ER α with GRIP1, the GRIP1 LXXAA mutant, and the LXXLL peptides, dissociation by 10⁻⁶ M ICI 182,780 was assessed after a 24-h treatment with 10⁻⁹ M E₂. In contrast to the α / β V peptide, GRIP1 remained as tightly associated with ER α 4 h after ICI 182,780 addition as it was before ICI 182,780 addition (Fig. 8B). Complete resistance to ICI 182,780 challenge also was observed for E₂/ER/GRIP1 complexes formed after only 1 h of E₂ preincubation (not shown). The relative stability of the E₂/ER/GRIP1 complexes depended upon the integrity of

EXHIBIT C

the LXXLL sites as GRIP1 containing the LXXAA mutations was dispersed by ICI 182,780 addition in a time-dependent fashion (Fig. 8B). The E₂/ER/GRIP1 complexes similarly displayed an LXXLL-dependent resistance to challenge with 10⁻⁶ M raloxifene (not shown). These data suggested that interaction of the LXXLL motif with ER α might regulate the subsequent ligand access to ER α *in vivo* as suggested by *in vitro* studies (39). Indeed, ER α complexes formed with the isolated LXXLL peptides were also resistant to dispersal by ICI 182,780 (Fig. 8C), demonstrating that interaction with LXXLL alone was sufficient to alter the subsequent response of the preformed complexes to challenge with ligand.

DISCUSSION

We found that different ER ligands caused the recruitment of specific panels of nuclear receptor-interacting peptides and proteins to the intranuclear location occupied by ER α (Figs. 2, 5, and 6). The correlation of the ligand specificities of intranuclear recruitment (Figs. 2–7 and Table 1) with the previously reported ligand specificities of the direct interactions of GRIP1 and each peptide with ER α (6, 7, 12–14, 20) suggests that some colocalization involves direct interactions between ER α -BFP and the GFP-linked GRIP1 and target peptides. Consistent with direct interaction, simultaneous addition of an excess of antiestrogen also blocked intranuclear recruitment. Thus, recruitment in living cells faithfully reflected the known biochemical and molecular properties of well characterized ER-interacting factors.

In addition to confirming in living cells the ligand specificities of these previously known interactions, the analysis of intranuclear recruitment allowed us to follow complex formation with time after ligand addition. Temporal variations in both recruitment and dissociation were observed (Figs. 4, 5, 7, and 8). Temporally delayed recruitments may represent a secondary association of some complexes via intermediary factors that are initially recruited in response to ligand binding. Such an indirect interaction may be responsible for the delayed (Fig. 7) interaction of ER α with GRIP deleted of the two LXXLL motifs previously described (5) to be necessary for direct GRIP1 interaction with ER α . Alternatively, GRIP1 is known to interact with other regions of ER α (11), and the temporal delay of intranuclear recruitment of GRIP1 Δ LXXLL may result from time-dependent ER α interactions or modifications that may be required for the proper folding of these alternative GRIP1 interaction sites.

The molecular basis for the delayed recruitment to ER α of the isolated class II LXXLL (Fig. 4C) similarly remains to be defined. However, the direct interaction of the class II peptide with AF-2 in ER α is weak compared with the class I and class III LXXLL interactions (12), and the delayed intracellular recruitment may

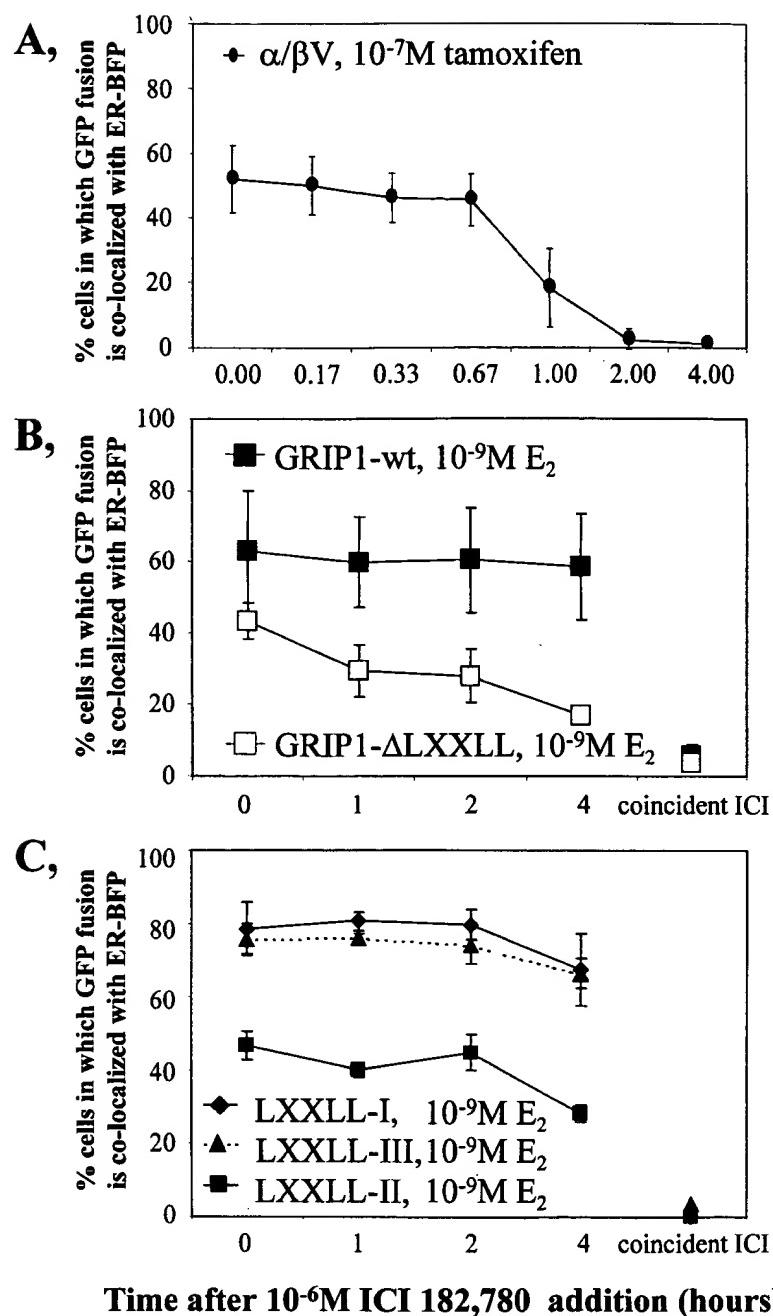


Fig. 8. Colocalized $E_2/ER\alpha/LXXLL$ Complexes Are Resistant to Dispersal by Antiestrogen

A, Colocalization of the non-LXXLL-containing GFP- $\alpha/\beta V$ peptide with ER α -BFP induced by $10^{-7} M$ tamoxifen is reversed by subsequent incubation with $10^{-6} M$ ICI 182,780. Complexes of ER α with GRIP1 (panel B) and each of the LXXLL peptides (panel C) induced by $10^{-9} M E_2$ are not disrupted even 4 h after the addition of $10^{-6} M$ ICI 182,780. In contrast, colocalized GRIP1- Δ LXXLL is readily dispersed from the intranuclear location of ER α upon ICI 182,780 addition. Coincident ICI, percent cells showing colocalization 24 h after simultaneous addition of $10^{-6} M$ ICI 182,780 and $10^{-8} M E_2$ in parallel experiments. Each graph represents the mean \pm SD in the percent of cells displaying colocalization from four independent experiments.

arise from class II LXXLL E_2 -induced associations with other regions of ER α , other ER-interacting factors, or even with ER α covalently modified by cofactors recruited to the E_2 -bound ER. Although the mechanisms for these temporal variations in intranuclear recruitment remain unknown, the previously unrecognized variations in the timing and sequence of complexes

recruited to ER after ligand addition are likely to be a key determinant of ligand response and adaptation and are uniquely detected by the intranuclear recruitment assay.

Challenge of the preformed complexes with competitive antagonists demonstrated that each peptide or cofactor was displaced from the complexes with

unique dissociation kinetics (Fig. 8). Complexes of ER α with LXXLL-containing peptides or cofactors were considerably more resistant to disruption by ICI 182,780 or raloxifene than complexes that did not involve LXXLL interactions, demonstrating that specific ligand-dependent interactions uniquely changed the nature of the complexes. The LXXLL-dependent reduction in the dissociation kinetics of the preformed complexes may be a consequence of the decreased off rate of ligand upon LXXLL binding to ER as recently reported *in vitro* (39). Alternatively, translocation of the LXXLL-containing peptide or GRIP1 from ER to another protein that resides in the same intranuclear position as ER could explain why the LXXLL-containing peptides and GRIP1 remain localized for prolonged periods of time after subsequent antagonist challenge.

The ligand specificity and temporal characteristics of intranuclear recruitment of ER α with each ligand indicated that, in the cellular environment, distinct conformations of ER are formed in response to E₂ and each SERM (7, 10). Recruitment was measured as a function of the percentage of cells responding to each ligand. The underlying basis for the cell-to-cell variability in recruitment remains to be described but may be responsible for the previous observation that dose-dependent transcriptional activation by a nuclear receptor ligand arises through an increase in the proportion of cells responding to the ligand rather than an equivalent, incremental increase in all cells (40).

The different abilities of E₂, tamoxifen, and raloxifene to promote ER α colocalization of the $\alpha/\beta\text{V}$ peptide, the class I LXXLL peptide, and GRIP1 provided dramatic evidence for the differing cellular and molecular properties of these clinically useful ligands. The E₂- and tamoxifen-induced recruitment of GRIP1 to the intranuclear location of ER α (Figs. 6 and 7) also contrasted with recruitment of related cofactor SRC-1a, which responded only to E₂, and not tamoxifen (27). This difference may be attributable to cell type or other experimental differences between laboratories, or to different ligand specificities for related cofactors in the context of the living cell. Nevertheless, the detection of these differences in living cells may prove useful in dissecting the differing clinical properties of E₂, tamoxifen, and raloxifene in different tissues (16, 18, 41, 42). The ability to quantify these changes on a pixel level (Fig. 4B) provide a first indicator that automated equipment can be developed for the high throughput measurement of ligand-specific effects on intranuclear recruitment and dissociation in living cells.

Recently, it was shown that the reticular intranuclear distribution of estrogen, SERM, and antiestrogen-bound ER α paralleled the tight binding of ER α to the nuclear matrix and that one ER-interacting factor, SRC-1a, was corecruited to the nuclear matrix via the ligand-bound ER (27). The results presented here suggest that other ER-interacting complexes may be similarly recruited to the nuclear matrix compartment upon ER binding to different ligands and that each

ligand promotes the recruitment of specific nuclear receptor-interacting peptides and proteins (Table 1) with unique temporal kinetics (Figs. 4, 5, and 7). The ligand-regulated association of ER and ER-interacting complexes with the nuclear matrix is intriguing given the historical association of transcription markers and enhancer/promoter sequences with the nuclear matrix (43, 44). The nuclear matrix may aid the organization of transcriptionally competent chromosomal domains (45) but a decisive correlation of nuclear matrix association with transcriptional activation or repression remains to be established (46).

Thus, we demonstrated that recruitment of ER-interacting factors to the intranuclear position of ER α is differentially regulated by the nature of the interacting sequence and the type of ligand. The complex temporal kinetics and ligand specificities of the association of ER α and its cofactors illustrated a variety of possible responses of ER α to ligand addition for which the intranuclear colocalization assay provided a direct read-out *in vivo*. Other methods currently used to detect ER-peptide or cofactor interactions rely on various *in vitro* binding assays or on two-hybrid assays in cells. The advantage of the intranuclear colocalization assay is that it is an *in vivo* assay in which direct and indirect interactions of ER with specific peptide or cofactor targets are readily measured in real time. Therefore, the intranuclear colocalization assay allows the intracellular actions of each ligand to be dissected in unprecedented detail. The availability of many more ER-interacting peptides and cofactors (6, 7, 12–14, 20, 47) will permit the detection of an even more expanded series of ER activities and may also facilitate the identification of novel ligands that induce specific subsets of cofactor interactions with ER or other nuclear receptors. Such novel ligands could be used to probe for the specific molecular events involved in nuclear receptor regulation of different genes and may even provide a rapid means for the identification of compounds with improved specificity for hormone replacement therapies.

MATERIALS AND METHODS

Expression Vectors

The cDNA encoding the BFP Y66H, Y145F variant of GFP (28) or the cDNA encoding RFP (CLONTECH Laboratories, Inc., Palo Alto, CA) were fused in frame to the carboxy terminus of human ER and placed under the control of the cytomegalovirus promoter in the previously described BFP expression vector (35). The EGFP cDNA (CLONTECH Laboratories, Inc.), modified to include the SV40 nuclear localization signal (NLS) at its carboxy terminus, was inserted into the pTRE "Tet-On" expression plasmid (CLONTECH Laboratories, Inc.). Because of its small size, the modified EGFP-NLS remained distributed throughout the cytoplasm and nucleus when expressed. LXXLL (12) and $\alpha/\beta\text{V}$ (13) ER-interacting peptides were fused in frame to the carboxy terminus of EGFP-NLS in the pTRE expression plasmid. The αII (13) peptide was fused to the carboxy terminus of EGFP-NLS in the pEGFP-C3 vec-

tor (CLONTECH Laboratories, Inc.). The representative class I, class II, and class III LXXLL peptide sequences are from the D2, D47, and F6 peptides isolated in Ref. 12. Native GRIP1 and GRIP1 containing mutations in which ER-interacting, LXXLL boxes II and III (5) were mutated to LXAA were appended to the carboxy terminus of GFP in the EGFP-C2 vector (CLONTECH Laboratories, Inc.).

Transfection

GHFT1–5 cells were grown in a 1:1 mixture of phenol red-free Ham's F12-DMEM containing estrogen-free 10% newborn calf serum. The cells were harvested and transfected by electroporation as described previously (35, 48) with 10 µg of the cytomegalovirus (CMV)-ER α -BFP or CMV-ER α -RFP expression vector, 10 µg of pEGFP-GRIP1 or pEGFP-GRIP1-ΔLXXLL, and 5 µg of pEGFP- α II or 3 µg of the pTRE-GFP-LXXLL or pTRE-GFP- α /βV expression vectors. pUHG17-1 (1.2 µg), which expresses the tetracycline repressor/VP16 activator (49) used to regulate expression of the pTRE plasmid, was cotransfected with the pTRE-GFP-LXXLL or pTRE-GFP- α /βV expression vectors. The transfected cells were plated onto coverslips and grown in estrogen-free media. Doxycycline (5 µg/ml) was added to the media to induce the Tet-On promoter except in Fig. 3B in which concentrations of doxycycline were varied from 0 to 15 µg/ml. One day after transfection, ER ligands were added at the indicated concentrations and imaged 24 h later (Figs. 2–6). For the E $_2$ time course experiments (Figs. 4C and 7), 10 $^{-8}$ M E $_2$ was added at the indicated time before imaging on the second day after transfection. For the ICI 182,780 antagonism time courses, cells were treated with 10 $^{-7}$ M tamoxifen (Fig. 8A) or 10 $^{-9}$ M E $_2$ (Fig. 8, B and C) for 24 h followed by addition of 10 $^{-6}$ M ICI 182,780 at the indicated times before imaging.

Microscopy and Image Analysis

After addition of ligand or ethanol control vehicle, fluorescence images from the transfected cells were acquired with a Axioplan microscope equipped with a 63 \times -oil immersion objective lens (Carl Zeiss, Thornwood, NY). Single-cell recordings of cells grown in chamber slides were obtained on an IX-70 inverted microscope (Olympus Corp., Lake Success, NY) and analyzed with Metamorph (Universal Imaging Corp., West Chester, PA) colocalization software. Dual color imaging using Hoechst and fluorescein isothiocyanate filter sets or GFP and rhodamine filter sets (Chroma Technology Corp., Brattleboro, VT) selectively distinguished blue from green fluorescence and green from red fluorescence, respectively. Appropriate controls in which ER α -BFP, ER α -RFP, or each GFP-peptide or GFP-GRIP were expressed individually ensured a lack of fluorescence bleedthrough between the channels. Grayscale images of the cells were obtained using a Xillix microscope (Carl Zeiss) or Hamamatsu ORCA microscope (Olympus Corp.) cooled CCD cameras. The digital images were background-subtracted and then converted to red-green-blue (RGB) images by assigning the GFP signal to the green channel, BFP signals to the blue channel, and RFP signals to the red channel of RGB digital images. Integration times and image processing were kept constant within each set of experiments.

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